Sensors & Actuators: B. Chemical 278 (2019) 140–146

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Development of a self-driving bioassay based on diffusion for simple detection of microorganisms

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ARTICLE INFO

Keywords:
Diffusion
Microparticles
Bacteria
Brownian motion
Self-driving bioassay

ABSTRACT

A self-driving, thermal-diffusion-based bioassay for the detection of microorganisms in a liquid medium is presented in this paper. In the bioassay, each particle functions as an individual sensing probe. Thus, the representative Brownian velocity of microparticles can be obtained by analyzing the velocity histogram of each particle. The ensemble average was used to enhance peak Brownian velocity. Relative error was reduced to 0.5% when the number of counted particles exceeded 60. The experimentally measured and theoretically derived Brownian velocities and diameters of the particles were in good agreement. The relative standard deviations of the temporal stability and reproducibility of the bioassay were maintained below 1.2%. A calibration curve was constructed and used to distinguish two mixed colloidal suspensions to provide proof that the bioassay can be used in practical applications. The particles were functionalized with antibodies to enable the real biological application of the bioassay in the capture and detection of motile *Pseudomonas aeruginosa* and nonmotile *Staphylococcus aureus*. The diffusivity values of both bacterial growth media decreased as bacterial concentration increased. Given that the viscosity of the growth media varied as bacteria proliferated, additional bacteria-free reference particles were added to the medium to provide dynamic background information. The diffusion-based bioassay presented here is easy to use, robust, and highly reliable. In contrast to most existing biosensors, it does not require an external power source and is thus ideal for use in resource-limited areas.

1. Introduction

Functionalized microparticles have been widely used in the detection of various analytes [1–4]. Although bead-based biosensing methods have high flexibility, sensitivity, and accuracy, their sensitivity, resolution, and reliability are mainly dependent on external readout equipment. The presence of target molecules is indicated by changes in the fluorescence or colors of dye-labeled microparticles [5–7]. Given that light intensity increases with the amount of target molecules, signals of the molecular activities can be quantified with photodetectors or simply interpreted visually. Wang et al. [8] proposed a novel optoelectrokinetic platform that enhances biosignals by concentrating fluorescent microparticles. In this platform, fluorescence is generated through the effect of Förster resonance energy transfer. The platform achieves a limit of detection of as low as 5 nM for the target protein lipocalin 1. In recent years, label-free methods have received considerable attention from the public because they enable the highly reliable quantification of target analytes. Kinnunen et al. [9] reported a novel method for determining the drug susceptibility of bacteria. Their reported method is based on an asynchronous magnetic particle rotation sensor driven by external electromagnetic coils. The magnetic particles were coated with vancomycin to capture free motile *Escherichia coli* cells. In this sensing system, particle rotation is altered by the surface attachment of bacteria. The alteration in particle rotation is terminated under the effect of antibiotic. In contrast to conventional antimicrobial susceptibility testing (AST), their method can determine the drug susceptibility of bacteria within 2 h. Zhao et al. [10] later developed a paper-based bioassay using DNA-modified gold nanoparticles deposited on paper substrates. The color of the paper substrate changes from blue to red when the dispersed gold nanoparticles aggregate in the presence of DNase I or adenosine. Qian et al. [11] attempted in vivo tumor targeting and detection by using surface-enhanced Raman (SERS) nanoparticle tags, which offer enhanced sensitivity, multiplexing, and quantification. The SERS nanoparticle

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https://doi.org/10.1016/j.snb.2018.09.087
Received 12 June 2018; Received in revised form 19 September 2018; Accepted 21 September 2018
Available online 26 September 2018

tags have been used successfully in the spectroscopic detection of small tumors (0.03 cm^3) with the penetration depth of 1–2 cm. Despite their advantages, however, the above methods require costly and sophisticated instruments for signal extraction. To eliminate the dependence of detection methods on external equipment, Gorti et al. [12] proposed bead-based diffusometry for viral detection. In this method, the diffusive state of microparticles in a medium could be monitored by measuring the width of a correlation peak obtained from the images of randomly moving particles. Diffusometric detection methods are failure-free because they rely solely on the self-driven Brownian motion of microparticles.

Diffusometric bioassays are more practical and suitable for real clinical applications than existing bioassays [13–18] because they do not depend on external power supplies and light intensity for detection. We have previously proven the feasibility of bead-based diffusometry for rapid AST [19,20], diabetic retinopathy diagnosis [21], and viscosity measurements [22,23]. In contrast to previous researchers who applied cross-correlation functions to characterize their diffusometric techniques, we characterized our technique by using the probability distribution function (PDF) of Brownian velocity measured over the duration of 33 s. Moreover, we evaluated the performance of our method on the basis of several parameters, including the number of images and data points, as well as magnification and stability. Notably, all microparticles were tracked individually in consecutive images. Then, we obtained the peak Brownian velocity to represent the diffusive state of all microparticles. Collective particle behaviors can be represented by statistical parameters because each microparticle functions as a self-driven biosensing probe. The results of our evaluations suggested that at least 80 data points are required to construct a meaningful velocity histogram, and the ensemble average of more than 60 particles is needed to obtain distinct Brownian velocities. The measured and predicted peak Brownian velocities of particles with different diameters were in good agreement. Temporal stability and reproducibility showed relative errors of less than 1%. Large and small particle sizes, however, resulted in parallel shifts away from predicted values because of the poor identification of particle displacement. We then successfully applied our approach in the identification of two colloidal suspensions containing particles with different sizes. Finally, we functionalized microparticles with antibodies to capture *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Both bacterial species could be detected using our bioassay in a dose-dependent manner. Notably, motile microorganisms may increase Brownian velocities by boosting microparticle diffusion. Nonetheless, diffusivity tended to decline with bacterial concentration. We counteracted background noise through the addition of reference microparticles to the same buffer medium. Our diffusion-based self-driving bioassay can provide a cornerstone for the rapid screening of other pathogenic microorganisms.

2. Methods and materials

2.1. Measurement system

The diffusometric platform comprised a fluorescent microscope (IX71, Olympus), a digital CMOS camera (FL3-U3-13S2C-CS, Point Grey Research Inc.), a computer, and a glass chip (Fig. 1A). The glass chip was simply constructed using a glass slide and a cover glass separated by a spacer (110 μm). A drop of analyte (5 μL) was pipetted on the glass chip for measurement. The microscope was equipped with a filter cube (U-MWIB3, BP460-495/BM505/BA510-IF, Olympus) and objectives with different powers (10×, 20×, and 40×) in accordance with the fluorescent microparticles used. The measurement plane was focused on the central depth of the glass chip to avoid impeding diffusion. To acquire images with good quality, the digital camera was set at a frame rate of 15 Hz in color mode. A total of 500 image frames were recorded for the duration of 33 s for each measurement. Although the system did not need to be set up on a vibration-free table, interferences from nearby fans or experiments on the same table were avoided. The laboratory temperature was controlled at 24 °C with an air conditioner. Slight variations in medium temperature and viscosity can be cancelled out through the addition of reference microparticles. In the absence of reference microparticles, however, the relative error due to the temperature variation of ± 1 °C was estimated to be less than 0.6%.

2.2. Diffusion-based algorithm

The Stokes–Einstein relation [24] states that diffusivity is inversely proportional to particle diameter when all other parameters are controlled. Langevin's theory is used to describe the relationship between time-averaged Brownian velocity and diffusivity [25]. Each functionalized microparticle can be used as a biosensing probe by simply tracking its Brownian velocity over time (Fig. 1B). The Brownian velocities of numerous particles are needed to construct a histogram that provides meaningful information. Data points were fitted with a six-order polynomial curve, and peak velocity was derived from the first-order derivatives of the curve. Through ensemble average with numerous velocity histograms, the diffusion-based algorithm was stabilized, and the effects of background fluctuations were avoided. The velocity histogram was created by sorting Brownian velocities within the measurement duration (500 frames at 15 Hz). All individual particles were tracked by using the ImageJ (https://fiji.sc/) plug-in program PTA2 (https://github.com/arayoshipta/PTA2). Image processing is described in detail in the Supplementary Information section. The maximum point of the polynomial curve was taken as the representative Brownian velocity for the diffusive state of a selected particle. In principle, the peak Brownian velocity of a small particle is larger than that of a large particle. Given that Brownian velocity is sensitive to the change in the size of each microparticle, in this study, microparticles were functionalized with antibodies to capture specific microorganisms. Although particle shape may alter Brownian velocity, variations among microparticles can be averaged out through the addition of reference microparticles.

The viscosity and temperature of a complicated medium, such as biological buffers, may slightly vary with time because of inhomogeneity in contents. To alleviate variations, reference particles were added to the same medium to provide information on background fluctuations. Thus, the relative Brownian velocity of a single microparticle can be expressed as

$$\frac{S}{S_0} = \frac{\sqrt{d_{p0}}}{\sqrt{d_p}} \tag{1}$$

where d_{p0} and d_p represent the diameters of the reference and probe particles, respectively. The relative Brownian velocity is only associated with the change in particle size and is unaffected by other disturbances, i.e., changes in viscosity and temperature.

2.3. Reagents and microorganisms

Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (N), N-hydroxysuccinimide (NHS), 2-(n-morpholino)-ethanesulfonic acid (MES), and gentamicin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-*P. aeruginosa* polyclonal antibody (Ab, ab67905), anti-*S. aureus* polyclonal Ab (ab20920), and anti-TNF-α monoclonal Ab (ab9348) were acquired from Abcam (Cambridge, MA, USA). Tryptic soy broth (TSB) was obtained from BD (East Rutherford, NJ, USA).

2.4. Microparticle functionalization

Four plain fluorescent polystyrene (PS) particles with sizes of 0.5, 1, 3.2, and 4.8 μm (G500/R0100/R0300/G0500, 1% solids, Thermo